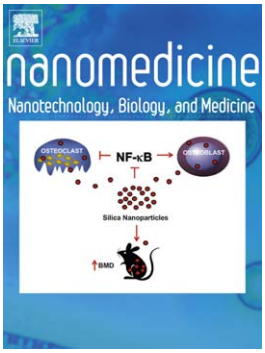



# Pharmacodynamic and pharmacogenomic study of the nanoparticle conjugate of camptothecin CRLX101 for the treatment of cancer

Shikha Gaur PhD, Yafan Wang, Leo Kretzner, Linling Chen, Terence Yen, Xiwei Wu PhD, Yate-Ching Yuan PhD, Mark Davis PhD, Yun Yen MD, PhD

PII: S1549-9634(14)00186-5
DOI: doi: 10.1016/j.nano.2014.04.003
Reference: NANO 930

To appear in: *Nanomedicine: Nanotechnology, Biology, and Medicine*

Received date: 3 January 2014
Revised date: 7 April 2014
Accepted date: 10 April 2014

Please cite this article as: Gaur Shikha, Wang Yafan, Kretzner Leo, Chen Linling, Yen Terence, Wu Xiwei, Yuan Yate-Ching, Davis Mark, Yen Yun, Pharmacodynamic and pharmacogenomic study of the nanoparticle conjugate of camptothecin CRLX101 for the treatment of cancer, *Nanomedicine: Nanotechnology, Biology, and Medicine* (2014), doi: 10.1016/j.nano.2014.04.003

# Pharmacodynamic and Pharmacogenomic study of the nanoparticle conjugate of camptothecin CRLX101 for the treatment of cancer

Shikha Gaur, PhD[1], Yafan Wang[2], Leo Kretzner[1], Linling Chen[2], Terence Yen[1], Xiwei Wu PhD[3], Yate-Ching Yuan, PhD[3] , Mark Davis, PhD[4], and Yun Yen, MD , PhD [1,5,]*

**Author Affiliations**: [1]Department of Molecular Pharmacology, [2]Translational Research Laboratory, [3] Bioinformatics Core Facility, Department of Molecular Medicine, Beckman Research Institute of the City of Hope, Duarte, CA 91010, [4]Chemical Engineering, California Institute of Technology, Pasadena, CA 91125, [5]Institute of Translational Medicine, Taipei Medical University, 250 Wu-Hsing Street, Taipei, Taiwan 110

**Short Title: Pharmacodynamics and Pharmacogenomics of CRLX101**

***Corresponding Author**: Yun Yen M.D., Ph.D., F.A.C.P.,
City of Hope Comprehensive Cancer Center,
Beckman Center, Room 4117
1500 E. Duarte Road, Duarte, CA 91010, (626) 359-8111 x 65707,
Email: yyen@coh.org

**Word Count:** Abstract-**150**, Body Text/Figure legends-**4993,** References-**49**, Figures**: 5**

**Disclosure of Potential Conflicts of Interest**: Dr. M.E. Davis is a consultant to and has stock in Cerulean Pharma Inc.

**Source of support for Research:** The funding for the study was provided by the City of Hope internal funds to Yun Yen.

**Abstract**

CRLX101 is a nanopharmaceutical consisting of cyclodextrin-based polymer molecule and camptothecin. The CRLX101 nanoparticle is designed to concentrate and slowly release camptothecin in tumors over an extended period of time. Tumor biopsy and blood samples collected from patients with advanced solid malignancies before and after CRLX101 treatment are subjected to immunohistochemistry and pharmacogenomics. The expression of Topoisomerase-1, Ki-67, CaIX, CD31 and VEGF decreased after CRLX101 treatment. The expressions of these proteins are inversely proportional with survival duration of the patients. The Drug Metabolism Enzymes and Transporters (DMET) array shows an allele frequency in patients similar to global populations with none of the SNPs associated with toxicity. The results suggest that the observed lower toxicity is not likely be due to different genotypes in SNPs. CRLX101 demonstrates a promising anti-tumor activity in heavily pre-treated or treatment-refractory solid tumor malignancies presumably by inhibition of proliferation and angiogenesis correlating with tumor growth inhibition.

Key Words: Camptothecin, Nanoparticle, Polymer conjugate, Topoisomerase 1, HIF-1α, Immunohistochemistry, Solid tumor

**Background**

Cancer represents a broad group of various diseases, all involving unregulated and progressive cell growth. Collectively they account for approximately 13% of all deaths each year worldwide.[1]

Camptothecin (CPT), an alkaloid extract from plants known as *Camptotheca acuminata*, exhibits an anti-cancer activity against several types of cancer cells and animal tumor models due, at least in part, to the inhibition of DNA topoisomerase I (Topo-1) and resulting cell death.[2-4] Topo-1 remains a highly attractive drug target because it is essential for basic cellular processes including DNA replication, recombination and transcription. It is particularly up-regulated in rapidly dividing tumor cells.[5] CPT inhibits the resealing of single-strand DNA breaks mediated by Topo-1. CPT is not used clinically because of its poor solubility and high systemic toxicity. In particular at physiologic pH, the active lactone ring of CPT hydrolyzes to yield the inactive carboxylate form that strongly binds to serum albumin, resulting in further driving the reaction towards the inactive form.[3] Numerous methods and modifications have been employed to enhance the pharmacokinetics and pharmacodynamics of this compound. For example, irinotecan and topotecan belong in the category of small molecule analogs of CPT that overcome some of the limitations of the parent drug demonstrated good antitumor activity, but their use remains limited by dose-limiting toxicities.[6]

CRLX101 (formerly known as IT-101) is a nanoparticulate conjugate of 20(S)-camptothecin (CPT) and a cyclodextrin-based polymer.[7] In CRLX101, CPT is linked covalently through a glycine linker to the linear copolymer CDP, which in turn consists of alternating subunits of beta-cyclodextrin and polyethylene glycol (PEG).[7] Conjugation of CPT to CDP increases the aqueous solubility of active CPT by three orders of

magnitude over native CPT and prevents inactivation through spontaneous lactone ring opening at physiologic pH. CRLX101 is preferentially accumulated in tumors due to leaky vasculature known as enhanced permeability and retention (EPR) effect, and slowly released inside tumor cells as the linkage is hydrolyzed, thereby reducing systemic exposure and the subsequent toxic side effects.[8,9] Preclinical studies have shown that sustained release of active CPT from CRLX101 nanoparticles results in extended Topo-1 and HIF-1α inhibition, thus increasing antitumor efficacy, as compared to standard dosing for irinotecan or topotecan.[8,10-14] Results from several studies have shown presence of CRLX101 as intact nanoparticles in tumor sections from both animals and patients.[11,13,15,16]

Human cancers are characterized by intratumoral hypoxia that results from deregulated cell proliferation. Physiological responses triggered by hypoxia affect all critical aspects of cancer progression, including immortalization, transformation, differentiation, genetic stability, angiogenesis, invasion, metastasis, treatment outcome and resistance to therapy.[17] Expression of carbonic anhydrase IX (CaIX), a member of the carbonic anhydrase family that catalyzes the reversible hydration of carbon dioxide to carbonic acid, is directly driven by hypoxia inducible factor-1α (HIF-1α).[18] This gene product is expressed in many types of human cancers and is usually absent in their normal counterparts and is linked with poor prognosis in several tumor types.[18,19] Camptothecin and its analogs have been reported to function by not only inhibiting HIF-1α transcriptional activity but also protein accumulation.[20] HIF-1α is known to up-regulate genes involved in a variety of processes, including angiogenesis.[19] Vascular endothelial growth factor (VEGF) and cluster of differentiation 31 (CD31) have been examined widely as biomarkers for angiogenesis and are shown to correlate with stage, grade and prognosis of patients.[21,22] A significant decrease in expressions of Ki-67, CaIX,

VEGF, and CD31 proteins in CRLX101 treated tumors was also observed in mice by immunohistochemistry (IHC) indicating an inhibition of proliferation, HIF-1α and angiogenesis.[11]

Another challenge of the present day clinical research is to assess the least toxic and most effective therapy for a specific patient because of the genetic variations encoding for metabolizing enzymes and drug transporters, which have been demonstrated to affect interindividual drug responses by alteration of absorption, distribution, metabolism and elimination (ADME).[23] The Affymetrix Drug Metabolism Enzymes and Transporters (DMET) microarray is the first assay enabling the simultaneous genotyping of a large number of known markers (1,936 markers in 225 genes) in drug ADME.

CRLX101 is currently under investigation in several human trials (**Clinical Trials. Gov Identifier:** NCT01380769, NCT01652079, NCT01625936 and NCT01612546). We report here pharmacodynamic and pharmacogenomic responses of CRLX101 in patient samples enrolled in the phase1/2a clinical trial. We further investigated whether CRLX101 may demonstrate a different DMET profile in comparison to historical controls.

## Methods

### Materials

CRLX101 was obtained from Cerulean Pharma Inc. (Cambridge, MA). Antibodies against different proteins were purchased from Cell Marque (Rocklin, CA), Novus Biologicals (Littleton, CO), R&D Systems Inc. (Minneapolis, MN) or Spring Biosciences (Pleasanton, CA). PAXgene tubes were purchased from Becton, Dickinson (Franklin Lake, NJ). Quantitative RT-PCR was performed using kits from Qiagen (Valencia, CA) and life Technologies (Carlsbad, CA). DMET array was purchased from Affymetrix (Santa Clara, CA). Human Cytokine Thirty-Plex Antibody Bead Kit was purchased from Invitrogen (Carlsbad, CA). All other reagents were obtained from Fisher Scientific (Pittsburgh, PA).

### Patient selection and study design

Patients included in the study were ≥ 18 years old with histologically or cytologically confirmed metastatic or unresectable solid tumor malignancies refractory to standard curative therapy. Additional eligibility requirements were as described in Weiss et al.[16] This trial was conducted at City of Hope Comprehensive Cancer Center with the approval of the Clinical Protocol Review and Monitoring Committee and the Institutional Review Board. This was an open label, single arm, dose escalation phase 1/2a study of CRLX101 (2a being an amended protocol after determination of maximum tolerated dose (MTD) with additional patient enrollment in MTD expansion cohort). Intravenous CRLX101 (6,12,15 or 18 mg/m$^2$) was infused over a 60-minute period on days 1, 8, and 15 of each 28-day cycle (weekly schedule) in the early part of the phase 1 trial and subsequently, on days 1 and 15 of each 28-day cycle (bi-weekly schedule). CRLX101

treatment continued until disease progression (determined by Response Evaluation Criteria in Solid Tumors [RECIST] version 1.1), patient withdrawal, or excessive toxicity.[16] Patient tumor samples were collected on 2-3 days prior to CRLX101 treatment cycle 1(pre-treatment) while another biopsy sample was collected from one patient 3 days after day 8 infusion of 2nd cycle post CRLX101 treatment (post-treatment). Blood samples were collected on day 1 of treatment prior to the initial dose and on day 8 and 15 post CRLX101 treatment.

**Tissue preparation and immunohistochemical staining**

The immunohistochemical assays were performed using a Dako Autostainer Plus (Dako Colorado Inc., CO) with fresh sections of tumor biopsy tissue removed before and/or after the treatment as described in .[11] Primary rabbit monoclonal Topo-1 (Abcam, MA), rabbit Ki-67 (Novus Biologicals, CO), goat CaIX (R&D Systems Inc., MN), rabbit polyclonal VEGF (Spring Biosciences, CA) or mouse monoclonal CD31 antibody (Cell Marque, CA) was applied for 1 – 2 hours at room temperature (20–25°C) at a final concentration of 1:100 (Topo-1 and Ki-67), 1:50 (CaIX), 1:500 (VEGF) or 1:75 (CD31). Antigen detection was accomplished using the vectastain elite ABC goat HRP kit (Vector labs, CA). The sections were counterstained with Meyer's hematoxylin and each run also included phosphate buffered saline (PBS) replacing the primary antibody for the negative controls, whereas samples known to express Topo-1, Ki-67, CaIX, VEGF, or CD31 served as positive controls. Photomicrographs were taken on a Nikon microscope equipped with a CCD camera (QImaging, Canada). Expression of proteins was quantified using a visual grading (0-no staining to 3-maximum staining) system on the basis of the extent of staining intensity (e.g., integrated optical density), and/or percentage of stained cells (e.g., total area or percentage of cells positive) for each image.

**RNA extraction and quantitative reverse transcription-PCR (RT-PCR)**

Peripheral blood was drawn in PAXgene tubes and total cellular RNA was isolated from whole blood using the PAXgene Blood RNA kit (Qiagen, CA). On-column DNaseI digestion was performed during RNA purification to remove Genomic DNA contamination. The RNA concentration and A260:A280 ratio was measured using a Nanodrop spectrophotometer (NanoDrop Technologies Inc., DE). Subsequent cDNA synthesis was carried out with Invitrogen SuperScript III reagents, with 2 μg total RNA + 5 ng/μL random hexamer primers followed by qRT-PCR in the ABI Prism Sequence Detection System (Applied Biosystems, CA). Reactions were run for 40 cycles of 95°C and 60°C alternation, for 15 and 30 seconds, respectively. Relative mRNA expression levels were calculated using the $\Delta C_T$ method against β-actin. The primer pair used is as follows:

Topo-1 Forward: 5'-TGC AAA AAT AAA GAA GGA GAA GGA A - 3'

Topo-1 Reverse: 5'-CTC TTT AGG AAC AAA ATA GCC AT – 3'

**Exploratory Biomarker Analyses:**

Plasma samples were analyzed using a Human Cytokine Thirty-Plex Antibody Bead Kit (Invitrogen, CA) as per the manufacturer's protocol using the Luminex 100 Flexmap 3D instrument (Luminex, TX) in the Clinical Immunobiology Correlative Studies Core Laboratory at Beckman Research Institute, City of Hope. Briefly, the blood was collected in endotoxin free tubes and centrifuged at 1000xg for 10 minutes. The ELISA assay was performed using plasma diluted with assay diluents and run along with the standards provided in the kit. Cytokine concentrations were calculated using Bio-Plex Manager 5.0 software with a 5-parameter curve-fitting algorithm applied to standard curve calculations of duplicate samples.

**DMET array:**

Genomic DNA from blood samples collected in PAXgene tubes was extracted using standard phenol/chloroform/isoamyl alcohol method from patients before and after CRLX101 treatment. Amplification, labeling and hybridization to DMET arrays were performed following Affymetrix's default protocol. Hybridized DMET arrays were washed and stained in the fluidic stations and scanned with the GeneChip® Scanner 3000 7G. Genotyping call rate and concordance comparisons were analyzed using the DMET Console (version 1.2) software. Fixed Genotype Boundaries was used as the algorithm for all genotyping configurations. Samples with QC call rate less than 90% were excluded.

Allele frequencies of variants on DMET array in the general population were obtained from Affymetrix web site, which included individuals represented from different regions of the world. The allele frequencies of these variants among the 14 patients were then compared to those among the global population. Fisher's exact test was used to identify the variants showing different allele frequency patterns from the population, with nominal p value cutoff < 0.01.

**Statistical Analysis:**

Graph Pad Prism 6.0 was used for statistical analysis and figure drawing. Group comparisons were performed using Student's *t*-test or two-way ANOVA and mean±standard error of the mean were used to present continuous variables. A p-value <0.05 was considered statistically significant. Kaplan–Meier analysis was applied to overall survival analysis. The Pearson's correlation coefficient analyzed the association between quantitative variables.

**Results**

Clinical characteristics and administered dosage of CRLX101 in patients included in study are summarized in Table S1.

**CRLX101 inhibits topoisomerase expression**

We checked the expression level of Topo-1 in pre- and post- CRLX101-treated skin biopsy samples from a triple negative breast cancer patient. The pre-treatment biopsy sample was collected 3 days prior to the start of first cycle while the post-treatment sample was collected 3 days after the day 15 infusion of cycle 2 of CRLX101 (5 infusions of CRLX101). Figure 1A (i) shows a strong nuclear expression of the enzyme in a majority of cells. This expression was inhibited in the post-CRLX101 treated tumor section Figure 1A (ii). Due to the difficulties of obtaining biopsy samples to measure the activity or expression of Topo-1 or various other genes in pre- and post-CRLX101 treatment group, we measure the mRNA expression levels in blood samples. The patients were divided based on Topo-1 expression level and were assigned a score of 0 (expression at day 8 less than expression at day 0) or 1 (expression at day 8 higher than day 0). Our results showed a pattern of decreased Topo-1 expression associated with longer survival duration (average survival duration fivefold in group with score of 1 compared to score 0 group) at day 8 post-CRLX101 treatment (Figure 1B). This did not reach statistical significance possibly due to the small number of samples ($p=0.07$). In order to see if there is any correlation between the survival time of these patients and pre- and post- CRLX101 treatment expression of Topo-1, we divided the total number of patients in two groups based on their survival duration. The mean survival time in group 1 ($\leq$ 6months, n=16) was 3.75±0.43 and group 2 ($>$ 6 Months, n=9) was 19.33±4.23 months. The survival duration was significantly different between the two groups

($p<0.005$). The pre-treatment samples showed a significantly higher expression of Topo-1 expression in group 1 as compared to group 2 ($p<0.05$, Figure 1C). The expression of Topo-1 showed an insignificant increase at day 8 before it came down at day 15 in group 1 patient samples while group 2 samples showed a decrease in the expression at day 8 as well as 15 after CRLX101 treatment (Figure 1D). These results coincide with an earlier report in which the immunohistochemical (IHC) staining in the nucleus of ovarian cancer cells isolated from ascites fluid two days following treatment of CRLX101 showed a 30% decrease in Topo-1 expression compared to pre-treatment levels and unwinding activity in tumor cells that was lower on day 2 following treatment compared with activity prior to treatment or on day 25 following treatment.[16]

**CRLX101 inhibits tumor cell proliferation and angiogenesis**

In an attempt to understand the effect of CRLX101 in pre- and post-treatment samples, the tumor sections were also processed for immunohistochemical expression of Ki-67, CaIX, VEGF and CD31. Ki-67 antigen is the prototypical cell cycle–related nuclear protein expressed by proliferating cells in all phases of the active cell cycle and absent in resting cells and is routinely used as a marker for proliferating cells. Cells in the Ki-67–immunostained section obtained from pre-treated tumor showed intense brown staining in the nuclear region. Representative photomicrographs of Ki-67 antigen–stained sections from pre and post-CRLX101 treated, tumors are shown in Figure 2A&B (i). The IHC reveals a significant decrease in Ki-67 staining in the post CRLX101 treated section as compared to pretreated tumor.

CaIX, which is associated with dysplasia and malignant tumors showed a higher expression of (100% cells positive for CaIX) in the pretreated section Figure 2A (ii) as compared to the CRLX101 post-treatment tumor section Figure 2B (ii).

Since angiogenesis is crucial for tumor development, CD31 has been widely used as a marker to highlight the density of intratumoral vessels and the degree of neoangiogenesis. Based on CD31 staining, the number of vasculature structures was significantly more in the pre-treatment tumor section as compared to post–treatment section Figure 2A & B (iii). VEGF is considered to be another important, directly acting and potent angiogenic agent over-expressed in tumors. The results reveal a higher expression of cytoplasmic localization of VEGF in the pre-treatment tumor section (Figure 2A iv). The expression was reduced in the CRLX101 post-treatment tumor section (Figure 2B iv). Figure 2 A &B (v) show the H & E staining in pre- and post-tumors sections.

We next compared the IHC expression scores of Topo- 1, Ki-67, CaIX, CD31 and VEGF in pre-treatment biopsy tumor sections in representative patients enrolled in the study. The expression level of all proteins was higher in tumor from the patient surviving 3 months as compared to tissue from a patient who survived 27 months (Figure 3A (i-v). Figure 3A (vi) show the H & E staining in tumor sections from both patients. Although, the average staining intensity score for Topo-1, Ki-67, CaIX and CD31 was also higher in group 1 as compared to group 2 (Figure 3B, i-v), it was significantly ($p<0.05$) different for only Topo-1 and Ki-67 expression. The expression of all the proteins was inversely proportional to survival duration of the patients (Figure 3C, i-v). The expression score for Ki-67 was best correlated ($p<0.01$), followed by Topo-1 and CaIX being next ($p=0.01$) respectively. The expression of CD31 and VEGF correlated the least with the survival time ($p<0.05$ and $p=0.27$). The expression score for Ki-67 when combined with another variable such as either of CaIX, CD31 or Topo-1 resulted in a significant increase in the correlation coefficient ($p<0.01$) respectively with survival time.

These data suggest that the combined score of two variables may be used for predicting the outcome of the treatment.

**Plasma Cytokine and Platelet Levels**

Elevated plasma cytokine levels have been reported in different types of cancers and sometimes correlated with increased side effects of the treatment and disease progression. There is a burgeoning amount of evidence, particularly in breast and prostate cancer, that markers of increased immune inflammatory activity are associated with fatigue.[24] Although the clinical meanings of the relationships is not yet known, the increase in levels of cytokines likely contributes to the symptoms of asthenia, fatigue and lethargy, as supported by cytokine-induced sickness behavior in animal models and humans.[25,26] The most common CRLX101 related adverse effect in patients included fatigue, anemia, neutropenia, leucopenia and thrombocytopenia. To better understand causes of fatigue, we assessed plasma cytokine levels (listed in Figure 4) prior to treatment and on day 8 of cycle 1 of CRLX101 treatment. Majority of these cytokines were already at levels several fold higher than the normal range in all the patients and mean plasma levels of most of these cytokines increased further although not significant, with CRLX101 treatment. The survival duration was longer for patients with lower IL levels as compared to the patients with shorter survival duration (Table S2). Our results showed that IL2R, the receptor for IL-2 levels expression was almost 2X higher for group 1 than group 2 (715±138 vs 350±50pg/ml, $p<0.05$). This is in agreement with a report showing a significantly higher IL2R expression in late stage lung cancer as compared to early stage colorectal cancer.[27] Similarly, expression for IL-8 was several folds higher for the group 1 compared to group 2 (164±96 vs 30±5pg/ml). Although not significant, this level increased further after CRLX101 treatment in group 1 while decreasing in group 2.

These results are supported by a study an *in vivo* tumorigenesis analysis in pancreatic cancer showing that tumor tissues from patients with higher serum IL-8 levels grew faster than those with lower IL-8 levels.[28] The levels of MIP-1a and MCP-1 were higher in the longer surviving group and increased further after CRLX101 treatment. IFN-α also increased after CRLX-101 treatment. Additionally, while not statistically significant, only IL-1Ra, IL-10 and IL-12p40/p70 values were reduced after CRLX101 treatment. In patients having an adverse event (AE) grade of ≥ 3, higher percentage change in plasma cytokine levels were observed when compared to a patient with an AE of grade 1 (Figure 4A). Among all patients with AE of grade 3, only one patient was from the longer survival group. Our results are in agreement with previous reports shown in patients with breast and prostate cancer.[24,29] Other studies have also shown that high innate production capacity of proinflammatory cytokines is associated with increased tumor burden, aggressiveness and metastasis.[30,31]

Additionally, analysis of patient blood samples revealed that mean platelet counts were transiently decreased, on day 14 of cycle 1 compared with pre-treatment levels. This decrease was significant only in group 1 patients whose survival duration was shorter than 6 months ($p = .034$, Figure 4B). Platelet aggregation was also observed in some cases; however, neither the platelet number nor aggregation of platelets was associated with clotting or excessive bleeding in any patient. A significant decrease in hemoglobin in group 1 compared to group 2 (15% vs 3.5%) may also account for fatigue ($p= 0.05$, Figure 4C). Portenoy and Itri demonstrated an association between chemotherapy-induced mild-to-moderate anemia and both fatigue and quality-of-life impairment. They also showed that patients with an increase in hematocrit of >6% also demonstrated significant improvement in energy level and daily activities.[32]

**Response and genotyping analysis**

To exclude the possibility that the lower toxicity observed in these patients might be due to allele frequency difference in SNPs of drug metabolism genes, we compared the allele frequencies between the patient cohort in the current study (n=8, Group 1 and n=7, Group 2) and the global population data obtained from Affymetrix, which included 713 HapMap samples consisting of individuals from various descent. Figure 5A indicates that the frequencies of the alleles on DMET array in these patients are very similar to those of the population (Chi Square test $p > 0.05$). The enzyme activity of genes on the DMET array was also categorized with Affymetrix's Genotyping Console, and the association of these enzymes with toxicity and response phenotype was examined. Using Fisher's exact test ($P<0.01$), 157 SNPs were identified as having different allele frequency in patients compared to the general population. However, none of these SNPs were found to be associated with either toxicity phenotype or response status. The only SNP found to be overlapping with response association was a non-synonymous SNP in flavin containing mono-oxygenase 2 (FMO2) (Figure 5B). Although the sample size is small, these results suggest the observed lower toxicity in these patients was not likely due to different genotypes in these DMET SNPs. These results indicate that the lower toxicity in the current patient cohort is not due to allele frequency difference in drug metabolism genes.

**Discussion**

Numerous preclinical and clinical studies have demonstrated that treatment with Topo-1 inhibitors result in tumor regression.[6,11,33,34] It has also been suggested that the cytotoxic effects of these inhibitors are dependent on the stabilization of topoisomerase-DNA complex.[35] Both *in vitro* and *in vivo* studies have demonstrated that sensitivity to these agents is modulated by absolute value of Topo-1 levels and Topo-1 protein expression is correlated well with poor patient survival. Our results showed a significant decrease in the expression of Topo-1 in post treatment sections. This is in agreement with other studies showing a decrease in expression and activity of Topo-1 after treatment with camptothecin or its analogs.[16,35] Our results also showed significantly decreased expression of Topo-1 in patients with longer survival duration as compared to the group with shorter survival duration. These results are not supported by the earlier hypothesis that lower expression of Topo-1 is correlated with a worse response to Topo-1 inhibitors.[36,37] However, results of other studies from Dutch colorectal cancer group failed to replicate the findings and concluded that although absolute Topo-1 expression may play a role, it is likely that additional pathways contribute to irinotecan sensitivity of Topo-1.[38,39] Therefore, these results indicate that clinical significance of Topo-1 is still unknown and remains controversial as prognostic marker.

Our results showed a decrease in expression of Ki-67 post-CRLX101 treatment. Also, the expression of Ki-67 was higher in the tumor section of a patient who survived for 3 months as compared to the one with a longer survival time (27 months). Our results are in agreement with studies that suggested a positive correlation between Ki-67 score and shorter survival.[40,41] We have previously shown that CRLX101 treatment inhibited expression of CaIX in a tumor sections. Also the expression of CaIX was lower in the sample from the patient with longer survival duration as compared to the other. However,

mRNA expression of HIF-1α showed insignificant difference before and after treatment (data not shown). These results are consistent with earlier reports of inhibition of hypoxia by camptothecin analogs.[11,42,43] HIF-1α is known to upregulate genes involved in a variety of processes such as angiogenesis.[17] VEGF and CD31 have been examined widely as biomarkers for angiogenesis and are shown to correlate with stage, grade, and prognosis of patients.[21,22] Our results showed a decrease in expression of VEGF and CD31 (microvessel density) in the post-treatment tissue sample. Also, the tissue from the patient with a shorter duration of survival had stronger expression of both the proteins as compared to the tissue from the longer surviving patient. Therefore, we conclude that expression of Ki-67, CaIX, CD31 and VEGF are inversely proportional to patient's survival duration. These results are consistent with previous reports that increased proliferation, positive expression of HIF-1α and VEGF are associated with shorter disease-free survival and overall survival in patients with gastric cancer.[40,44]

High incidence of gastrointestinal toxicity, hemorrhagic cystisis and severe bone marrow suppression in early clinical trials dampened the usage of CPT and encouraged the development of CPT analogs .[45] Two less potent small molecule derivatives (topotecan and irinotecan) achieved notable improvement over CPT but are still not well tolerated.[46] Our phase 1/2a result showed CRLX101- related adverse events including fatigue, cystitis, anemia, neutropenia, leucopenia and thrombocytopenia in some patients, but these events have been far less in severity as compared to CPT or its derivatives.[16] The levels of most common interleukins increased after treatment with CRLX101; however, they were lower in the group whose survival time was longer than 6 months suggesting that secretion of cytokines might be used as a tool to monitor the therapeutic indices of the drug. Also, higher levels of MIP-1a in the longer surviving group and its increase after treatment with CRLX101 in both groups is in agreement with the report

that tumor cells transfected with the MIP-1a gene might be useful as an effective therapy for the treatment of certain tumors.[47] Low serum MCP-1 levels have been identified in patients with worse prognosis similar to that reported in the current study. Interferon has been shown to facilitate anti-tumor activity by promoting antigen presenting cell mediated expansion of cytotoxic T cells and activating macrophages to release molecules such as superoxide.[48] Our results also show an increase in IFN-α levels after CRLX-101 treatment. A phase Ib study of the hepatocyte growth factor (HGF)-inhibitor AMG-102 in combination with either bevacizumab or motesanib identified post-treatment elevation in HGF levels which coincided with a 75% increase in all grades of fatigue, similar to those reported in the current study.[49] This data suggests that high innate production capacity of proinflammatory cytokines is associated with shorter survival, probably because of increased tumor growth and metastasis. A significant decrease in platelet counts and hemoglobin by day 14 of the first treatment cycle may provide a possible mechanism for treatment-related fatigue; however, additional studies are needed to clarify the relationship.

The DMET array enables a cost-effective measurement of existing and new metabolic pathway involvement by providing broad coverage of relevant pharmacogenetic markers in one assay. However, due to the small sample size, the chi-square p value was not significant for the patient group and a larger cohort should be used to validate these results.

In summary, these results reinforce the statement of the importance of CRLX101 as a promoter of increased anti-tumor activity in patients with solid tumor malignancies. These results also confirm that the possible mechanistic rationale for treatment with CRLX101 in the patients is inhibition of proliferation, hypoxia and angiogenesis

correlating with slow tumor growth presumably due to lack of survival factors supplied through blood and therefore longer survival time.

**Acknowledgements**

We thank Ms. Sofia Loera for histopathological staining, Ms. Mariko Lee with microscope imaging, Dr. Lufen Chang for helpful suggestions, Dr. Xiyong Liu for statistical analysis, and Scott Eliasof, Edward Garmey and Andre Zahn for editing of the manuscript.

**References**


1. Dranitsaris G, Truter I, Lubbe MS, Amir E, Evans W. Advances in cancer therapeutics and patient access to new drugs. *Pharmacoeconomics*. 2011;**29**(3):213-224.
2. Hertzberg RP, Caranfa MJ, Hecht SM. On the mechanism of topoisomerase I inhibition by camptothecin: evidence for binding to an enzyme-DNA complex. *Biochemistry*. 1989;**28**(11):4629-4638.
3. Slichenmyer WJ, Rowinsky EK, Donehower RC, Kaufmann SH. The current status of camptothecin analogues as antitumor agents. *J Natl Cancer Inst*. 1993;**85**(4):271-291.
4. Kehrer DF, Soepenberg O, Loos WJ, Verweij J, Sparreboom A. Modulation of camptothecin analogs in the treatment of cancer: a review. *Anticancer Drugs*. 2001;**12**(2):89-105.
5. Husain I, Mohler JL, Seigler HF, Besterman JM. Elevation of topoisomerase I messenger RNA, protein, and catalytic activity in human tumors: demonstration of tumor-type specificity and implications for cancer chemotherapy. *Cancer Res*. 1994;**54**(2):539-546.
6. Houghton PJ, Cheshire PJ, Hallman JD, 2nd, Lutz L, Friedman HS, Danks MK, Houghton JA. Efficacy of topoisomerase I inhibitors, topotecan and irinotecan, administered at low dose levels in protracted schedules to mice bearing xenografts of human tumors. *Cancer Chemother Pharmacol*. 1995;**36**(5):393-403.

7. Cheng J, Khin KT, Jensen GS, Liu A, Davis ME. Synthesis of linear, beta-cyclodextrin-based polymers and their camptothecin conjugates. *Bioconjug Chem*. 2003;**14**(5):1007-1017.

8. Cheng J, Khin KT, Davis ME. Antitumor activity of beta-cyclodextrin polymer-camptothecin conjugates. *Mol Pharm*. 2004;**1**(3):183-193.

9. Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. *Cancer Res*. 1986;**46**(12 Pt 1):6387-6392.

10. Davis ME. Design and development of IT-101, a cyclodextrin-containing polymer conjugate of camptothecin. *Adv Drug Deliv Rev*. 2009;**61**(13):1189-1192.

11. Gaur S, Chen L, Yen T, Wang Y, Zhou B, Davis M, Yen Y. Preclinical study of the cyclodextrin-polymer conjugate of camptothecin CRLX101 for the treatment of gastric cancer. *Nanomedicine*. 2012;**8**(5):721-730.

12. Schluep T, Cheng J, Khin KT, Davis ME. Pharmacokinetics and biodistribution of the camptothecin-polymer conjugate IT-101 in rats and tumor-bearing mice. *Cancer Chemother Pharmacol*. 2006;**57**(5):654-662.

13. Schluep T, Hwang J, Hildebrandt IJ, Czernin J, Choi CH, Alabi CA, Mack BC, Davis ME. Pharmacokinetics and tumor dynamics of the nanoparticle IT-101 from PET imaging and tumor histological measurements. *Proc Natl Acad Sci U S A*. 2009;**106**(27):11394-11399.

14. Svenson S, Wolfgang M, Hwang J, Ryan J, Eliasof S. Preclinical to clinical development of the novel camptothecin nanopharmaceutical CRLX101. *J Control Release*. 2011;**153**(1):49-55.

15. Eliasof S, Lazarus D, Peters CG, Case RI, Cole RO, Hwang J, Schluep T, Chao J, Lin J, Yen Y, Han H, Wiley DT, Zuckerman JE, Davis ME. Correlating preclinical animal studies and human clinical trials of a multifunctional, polymeric nanoparticle. *Proc Natl Acad Sci U S A*. 2013;**110**(37):15127-15132.

16. Weiss GJ, Chao J, Neidhart JD, Ramanathan RK, Bassett D, Neidhart JA, Choi CH, Chow W, Chung V, Forman SJ, Garmey E, Hwang J, Kalinoski DL, Koczywas M, Longmate J, Melton RJ, Morgan R, Oliver J, Peterkin JJ, Ryan JL, Schluep T, Synold TW, Twardowski P, Davis ME, Yen Y. First-in-human phase 1/2a trial of CRLX101, a cyclodextrin-containing polymer-camptothecin nanopharmaceutical in patients with advanced solid tumor malignancies. *Invest New Drugs*. 2013;**31**(4):986-1000.

17. Stoeltzing O, McCarty MF, Wey JS, Fan F, Liu W, Belcheva A, Bucana CD, Semenza GL, Ellis LM. Role of hypoxia-inducible factor 1alpha in gastric cancer cell growth, angiogenesis, and vessel maturation. *J Natl Cancer Inst*. 2004;**96**(12):946-956.

18. Kato Y, Yashiro M, Noda S, Kashiwagi S, Matsuoka J, Fuyuhiro Y, Doi Y, Hirakawa K. Expression of a hypoxia-associated protein, carbonic anhydrase-9, correlates with malignant phenotypes of gastric carcinoma. *Digestion*. 2010;**82**(4):246-251.

19. Wykoff CC, Beasley NJ, Watson PH, Turner KJ, Pastorek J, Sibtain A, Wilson GD, Turley H, Talks KL, Maxwell PH, Pugh CW, Ratcliffe PJ, Harris AL. Hypoxia-inducible expression of tumor-associated carbonic anhydrases. *Cancer Res*. 2000;**60**(24):7075-7083.

20. Litvak DA, Papaconstantinou HT, Evers BM, Townsend CM, Jr. Targeting molecular pathways with camptothecin as novel therapy for gastric cancer. *J Gastrointest Surg*. 1999;**3**(6):618-624.

21. Inoue K, Ozeki Y, Suganuma T, Sugiura Y, Tanaka S. Vascular endothelial growth factor expression in primary esophageal squamous cell carcinoma. Association with angiogenesis and tumor progression. *Cancer*. 1997;**79**(2):206-213.

22. Maeda K, Chung YS, Ogawa Y, Takatsuka S, Kang SM, Ogawa M, Sawada T, Sowa M. Prognostic value of vascular endothelial growth factor expression in gastric carcinoma. *Cancer*. 1996;**77**(5):858-863.

23. Burmester JK, Sedova M, Shapero MH, Mansfield E. DMET microarray technology for pharmacogenomics-based personalized medicine. *Methods Mol Biol*. 2010;**632**:99-124.

24. Bower JE, Ganz PA, Tao ML, Hu W, Belin TR, Sepah S, Cole S, Aziz N. Inflammatory biomarkers and fatigue during radiation therapy for breast and prostate cancer. *Clin Cancer Res*. 2009;**15**(17):5534-5540.

25. Dantzer R. Cytokine-induced sickness behavior: mechanisms and implications. *Ann N Y Acad Sci*. 2001;**933**:222-234.

26. Eisenberger NI, Inagaki TK, Mashal NM, Irwin MR. Inflammation and social experience: an inflammatory challenge induces feelings of social disconnection in addition to depressed mood. *Brain Behav Immun*. 2010;**24**(4):558-563.

27. Wang X, Xu S, Lu G, Feng J, Zhou X, Zhu C. Clinical significance of examining IL-2R in peripheral blood lymphocytes of patients with gastric cancer. *Chin Med J (Engl)*. 2001;**114**(12):1320-1322.

28. Chen Y, Shi M, Yu GZ, Qin XR, Jin G, Chen P, Zhu MH. Interleukin-8, a promising predictor for prognosis of pancreatic cancer. *World J Gastroenterol*. 2012;**18**(10):1123-1129.

29. Seruga B, Zhang H, Bernstein LJ, Tannock IF. Cytokines and their relationship to the symptoms and outcome of cancer. *Nat Rev Cancer*. 2008;**8**(11):887-899.

30. Trompet S, de Craen AJ, Mooijaart S, Stott DJ, Ford I, Sattar N, Jukema W, Westendorp RG. High Innate Production Capacity of Proinflammatory Cytokines Increases Risk for Death from Cancer: Results of the PROSPER Study. *Clin Cancer Res*. 2009;**15**(24):7744-7748.

31. Plones T, Krohn A, Burger M, Veelken H, Passlick B, Muller-Quernheim J, Zissel G. Serum level of CC-chemokine ligand 18 is increased in patients with non-small-cell lung cancer and correlates with survival time in adenocarcinomas. *PLoS One*. 2012;**7**(7):e41746.

32. Portenoy RK, Itri LM. Cancer-related fatigue: guidelines for evaluation and management. *Oncologist*. 1999;**4**(1):1-10.

33. Morris R, Munkarah A. Alternate dosing schedules for topotecan in the treatment of recurrent ovarian cancer. *Oncologist*. 2002;**7 Suppl 5**:29-35.

34. Numbenjapon T, Wang J, Colcher D, Schluep T, Davis ME, Duringer J, Kretzner L, Yen Y, Forman SJ, Raubitschek A. Preclinical results of camptothecin-polymer conjugate (IT-101) in multiple human lymphoma xenograft models. *Clin Cancer Res*. 2009;**15**(13):4365-4373.

35. Gilbert DC, Chalmers AJ, El-Khamisy SF. Topoisomerase I inhibition in colorectal cancer: biomarkers and therapeutic targets. *Br J Cancer*. 2012;**106**(1):18-24.

36. Ikeguchi M, Arai Y, Maeta Y, Ashida K, Katano K, Wakatsuki T. Topoisomerase I expression in tumors as a biological marker for CPT-11 chemosensitivity in patients with colorectal cancer. *Surg Today*. 2011;**41**(9):1196-1199.

37. Horisberger K, Erben P, Muessle B, Woernle C, Stroebel P, Kaehler G, Wenz F, Hochhaus A, Post S, Willeke F, Hofheinz RD, Margit. Topoisomerase I expression correlates to response to neoadjuvant irinotecan-based chemoradiation in rectal cancer. *Anticancer Drugs*. 2009;**20**(6):519-524.

38. Koopman M, Antonini NF, Douma J, Wals J, Honkoop AH, Erdkamp FL, de Jong RS, Rodenburg CJ, Vreugdenhil G, Loosveld OJ, van Bochove A, Sinnige HA, Creemers GJ, Tesselaar ME, Slee PH, Werter MJ, Mol L, Dalesio O, Punt CJ. Sequential versus combination chemotherapy with capecitabine, irinotecan, and oxaliplatin in advanced colorectal cancer (CAIRO): a phase III randomised controlled trial. *Lancet*. 2007;**370**(9582):135-142.

39. Koopman M, Venderbosch S, van Tinteren H, Ligtenberg MJ, Nagtegaal I, Van Krieken JH, Punt CJ. Predictive and prognostic markers for the outcome of chemotherapy in advanced colorectal cancer, a retrospective analysis of the phase III randomised CAIRO study. *Eur J Cancer*. 2009;**45**(11):1999-2006.

40. Tan PH, Bay BH, Yip G, Selvarajan S, Tan P, Wu J, Lee CH, Li KB. Immunohistochemical detection of Ki67 in breast cancer correlates with transcriptional regulation of genes related to apoptosis and cell death. *Mod Pathol*. 2005;**18**(3):374-381.

41. Krishnamachary B, Glunde K, Wildes F, Mori N, Takagi T, Raman V, Bhujwalla ZM. Noninvasive detection of lentiviral-mediated choline kinase targeting in a human breast cancer xenograft. *Cancer Res*. 2009;**69**(8):3464-3471.

42. Rapisarda A, Uranchimeg B, Sordet O, Pommier Y, Shoemaker RH, Melillo G. Topoisomerase I-mediated inhibition of hypoxia-inducible factor 1: mechanism and therapeutic implications. *Cancer Res*. 2004;**64**(4):1475-1482.

43. Rapisarda A, Zalek J, Hollingshead M, Braunschweig T, Uranchimeg B, Bonomi CA, Borgel SD, Carter JP, Hewitt SM, Shoemaker RH, Melillo G. Schedule-dependent inhibition of hypoxia-inducible factor-1alpha protein accumulation, angiogenesis, and tumor growth by topotecan in U251-HRE glioblastoma xenografts. *Cancer Res*. 2004;**64**(19):6845-6848.

44. Fondevila C, Metges JP, Fuster J, Grau JJ, Palacin A, Castells A, Volant A, Pera M. p53 and VEGF expression are independent predictors of tumour recurrence and survival following curative resection of gastric cancer. *Br J Cancer*. 2004;**90**(1):206-215.

45. Creaven PJ, Allen LM, Muggia FM. Plasma camptothecin (NSC-100880) levels during a 5-day course of treatment: relation to dose and toxicity. *Cancer Chemother Rep*. 1972;**56**(5):573-578.

46. Ikegami T, Ha L, Arimori K, Latham P, Kobayashi K, Ceryak S, Matsuzaki Y, Bouscarel B. Intestinal alkalization as a possible preventive mechanism in irinotecan (CPT-11)-induced diarrhea. *Cancer Res*. 2002;**62**(1):179-187.

47. Nakashima E, Oya A, Kubota Y, Kanada N, Matsushita R, Takeda K, Ichimura F, Kuno K, Mukaida N, Hirose K, Nakanishi I, Ujiie T, Matsushima K. A candidate for cancer gene therapy: MIP-1 alpha gene transfer to an adenocarcinoma cell line reduced tumorigenicity and induced protective immunity in immunocompetent mice. *Pharm Res*. 1996;**13**(12):1896-1901.

48. Dehqanzada ZA, Storrer CE, Hueman MT, Foley RJ, Harris KA, Jama YH, Kao TC, Shriver CD, Ponniah S, Peoples GE. Correlations between serum monocyte chemotactic protein-1 levels, clinical prognostic factors, and HER-2/neu vaccine-related immunity in breast cancer patients. *Clin Cancer Res*. 2006;**12**(2):478-486.

49. Rosen PJ, Sweeney CJ, Park DJ, Beaupre DM, Deng H, Leitch IM, Shubhakar P, Zhu M, Oliner KS, Anderson A, Yee LK. A phase Ib study of AMG 102 in combination with bevacizumab or motesanib in patients with advanced solid tumors. *Clin Cancer Res*. 2010;**16**(9):2677-2687.

**Figure Legends**

**Figure 1**. **Effect of CRLX101 treatment on Topoisomerase expression: (A)**, Representative panel of immunohistochemical staining of Topo-1 in tumor sections extracted from a patient before and after treatment with CRLX101 (magnification 40x). (**B)**, The Kaplan-Meier analysis for overall survival on day 8 of CRLX101 treatment stratified by Topo-1 expression (from blood samples). (**C)** and **(D)**, Comparison of mRNA levels of Topo-1 determined by RT-PCR before and after CRLX101 treatment. Each sample was measured three times. The two groups were divided based on time of survival from date of CRLX101 administration.

**Figure 2**. Representative panel of immunohistochemical analysis of Ki-67, carbonic anhydrase (CaIX), cluster of differentiation molecule (CD31 also known as PECAM-1) and vascular endothelial growth factor (VEGF) in tumors extracted from a patient before (**A**) and after treatment (**B**) with CRLX101 (Same tumor tissue used for as in Figure 1A, Magnification 40x). The staining for all the proteins was strong for pretreatment section as compared to post treatment section; (**i**) The expression level of Ki-67 in nucleus of tumor cells; **(ii)** A membranous expression of CaIX in pretreatment, **(iii)** Expression of CD31 (cell junctions) and **(iv)** Cytoplasmic expression of VEGF (**v**) H&E staining of corresponding sections from untreated and treated tumors.

**Figure 3**. Representative panel of immunohistochemical analysis (A) Topo-1, Ki-67, carbonic anhydrase (CaIX), CD31 and vascular endothelial growth factor (VEGF) in tumors extracted from patients surviving 3 months and 27 months from the date of administration of CRLX101 (Magnification 40x). The staining for all the proteins (**i**) Topo-1, (**ii**) Ki-67, (**iii**) CaIX, (**iv**) CD31 and (**v**) VEGF were stronger for patient tumor section surviving 3 months as compared to patient surviving 27 months. (**iv**) H&E

staining of corresponding tumor sections from two patients. (**B i-v)** Histograms showing the difference in expression of Topo-1, Ki-67, CaIX, CD31 and VEGF (scoring based on IHC staining intensity) in two groups (n=4 each) divided based on survival duration ($p \leq 0.05$, represents a significant difference). **(C i-v**) Relationship between expression of proteins and survival duration from the time CRLX101 was administered in the patients with available biopsy sample.

**Figure 4**. Cytokine, platelet and hemoglobin analyses 8 days after treatment with CRLX101. (**A**) Comparison of percent change in plasma cytokine levels after CRLX101 treatment measured (in duplicate) by Thirty-Plex Antibody Bead Kit and calculated using Bio-Plex Manager 5.0 software with a 5-parameter curve-fitting algorithm applied for standard curve. The two groups were divided based on occurrence of adverse events of grade 3 and 1 in patients after treatment with CRLX101. (**B**) Platelet counts in blood samples from pretreatment and 15 day post CRLX101 treatment in two groups divided based on survival duration. Significant ($p<0.05$) decrease in platelet count observed in patients with shorter duration. (**C**) Hemoglobin levels in blood samples from pretreatment and 15day post CRLX101 treatment in two groups divided based on survival duration from time of drug administration. Significant ($p<0.05$) decrease in hemoglobin observed in patients with shorter duration.

**Figure 5**. Allele frequency analysis by DMET array: (**A**) Comparison of Allele Frequency in the Patients vs. the Population. Allele frequency in global populations was obtained from Affymetrix's annotation file (59 CEU, 90 CHB, 87 CHD, 91 JPT, 89 LWK, 60 MEX, and 119 YRI). (**B**) SNPs showing different allele frequency from Population associated with Response or Toxicity.

Figure 1

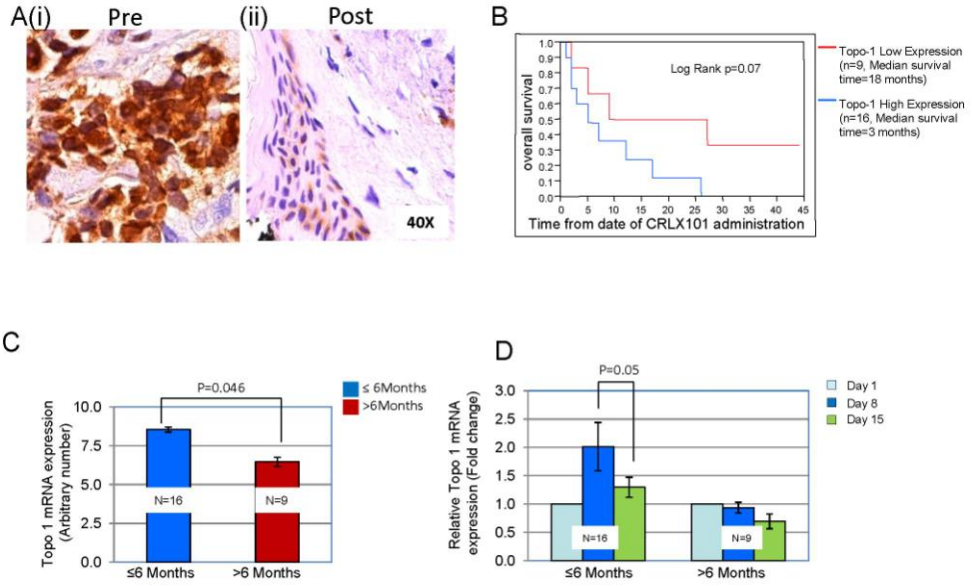



Figure 1

Figure 2

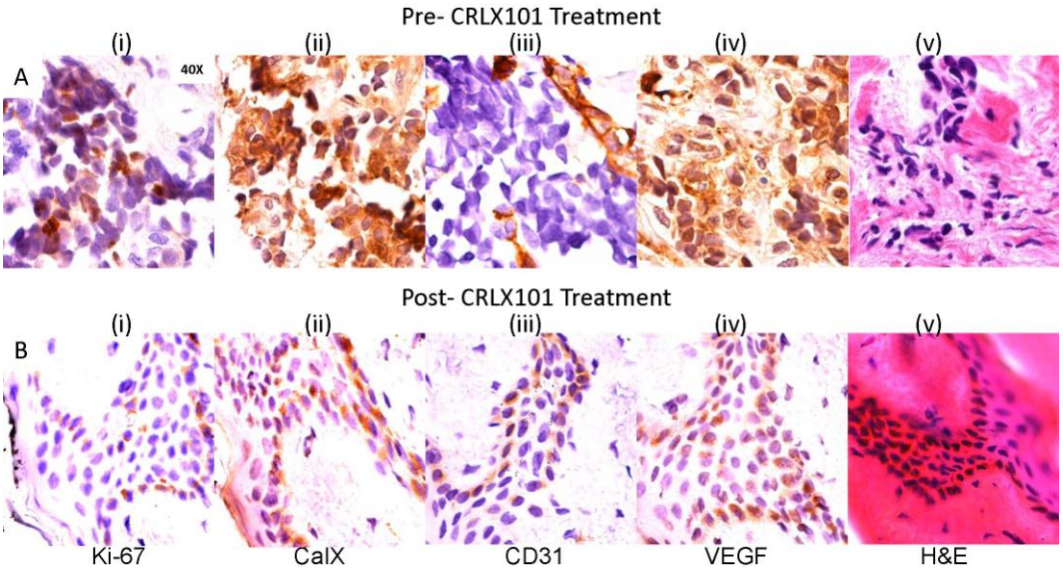



Figure 2

Figure 3

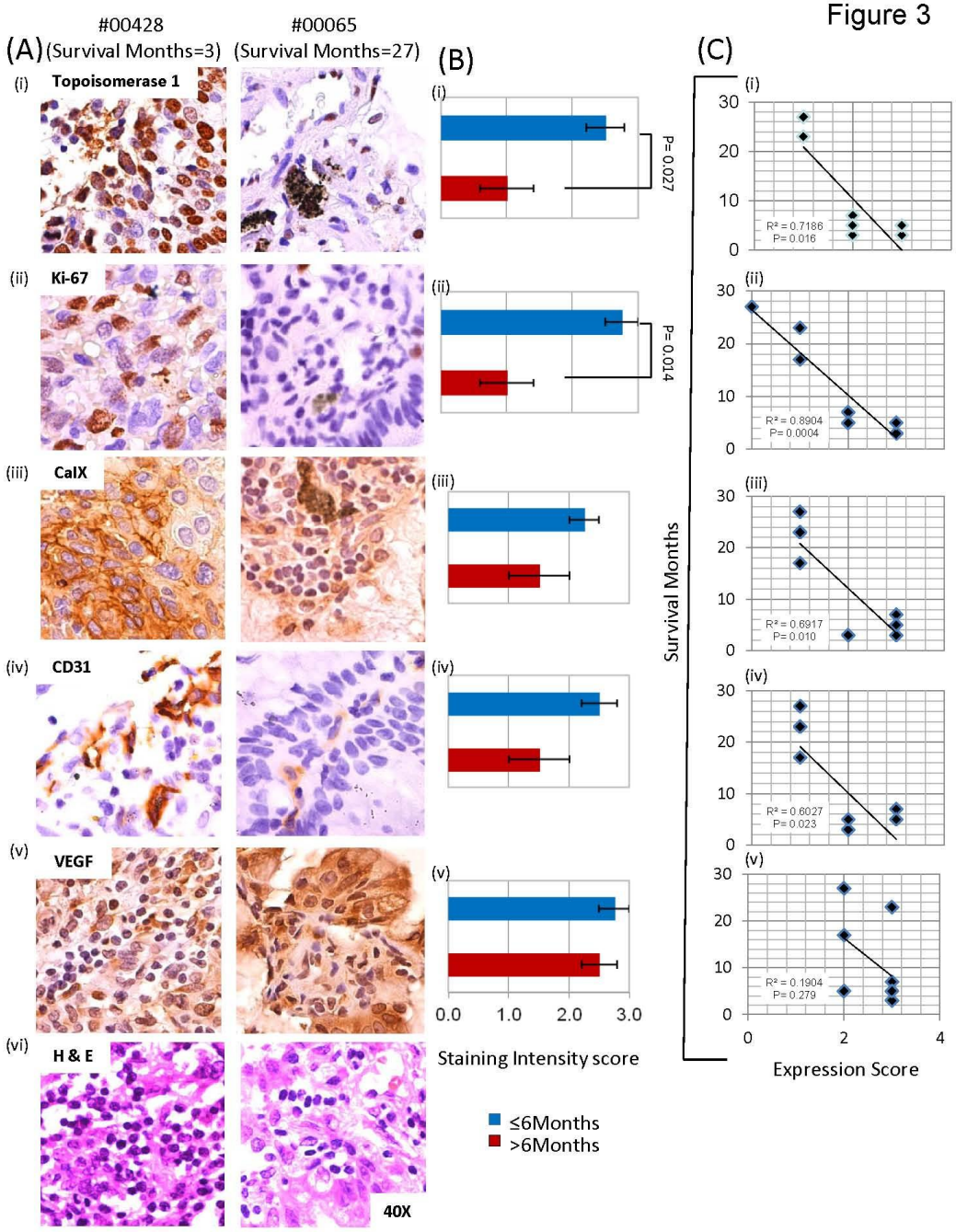



Figure 3

Figure 4

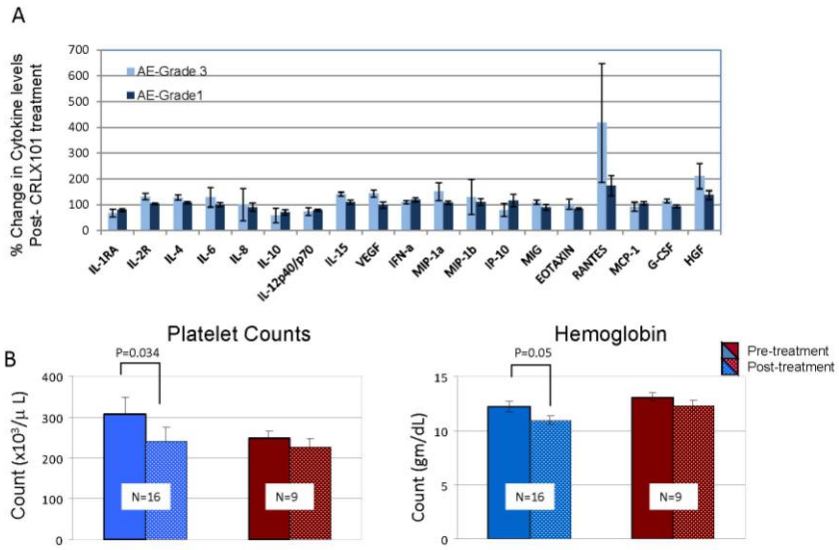



Figure 4

Figure 5

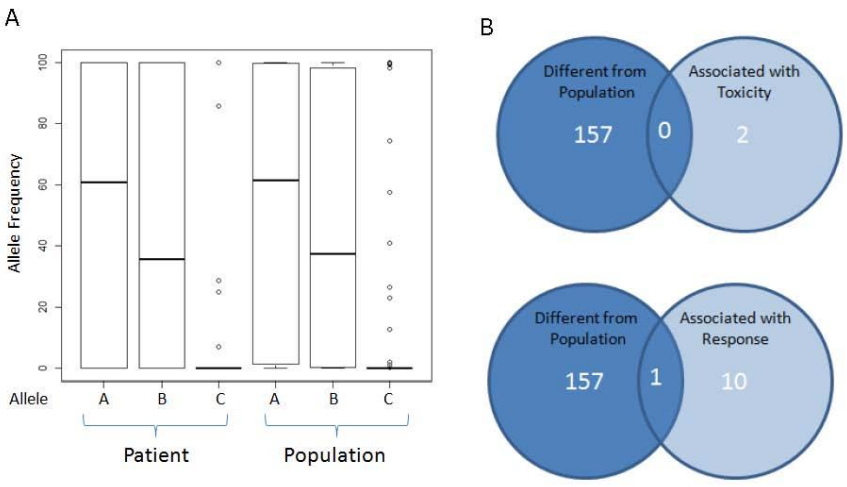
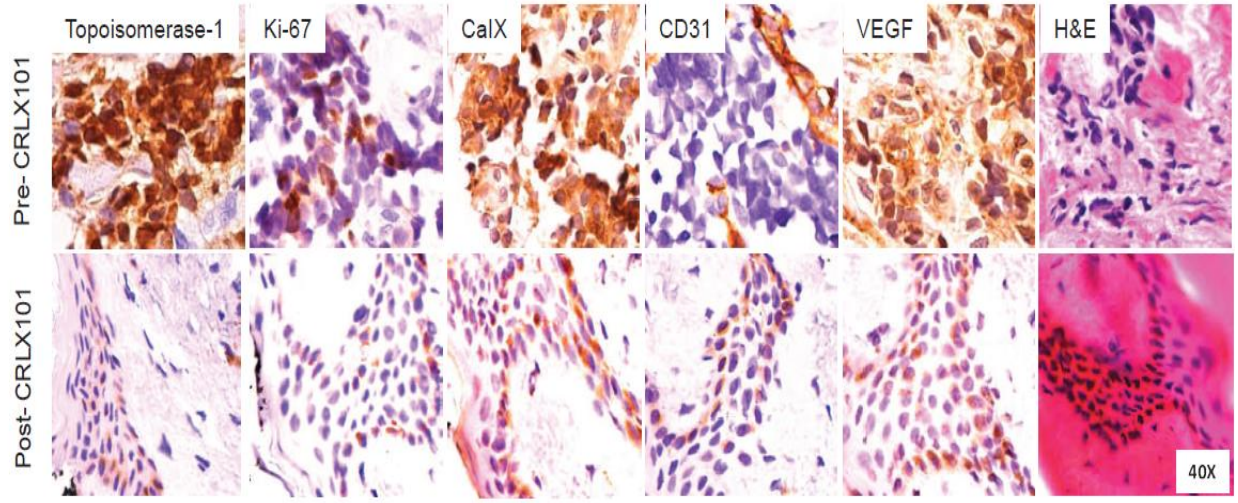



Figure 5

**Graphical Abstract**

The clinical efficacy of CRLX101, the nanoparticulate conjugate of cyclodextrin based polymer molecule and camptothecin is evaluated in patients with treatment refractory solid tumor malignancies by immunohistochemistry and pharmacogenomics. The decrease in expression of topoisomerase-1, Ki-67, CaIX, VEGF and CD31 after treatment with CRLX101 indicate inhibition of proliferation, hypoxia and angiogenesis correlating with slow tumor growth.




Graphical Image :The expressions of Topoisomerase-1, Ki-67, Ca IX, CD31 and VEGF decrease after CRLX101 treatment correlate with anti-tumor activity.